METALLOPROTEIN CROSSTALK: QUANTIFYING ZINC EXCHANGE BETWEEN STABLE ISOTOPICALLY LABELED PROTEINS USING DIRECTLY COUPLED HPLC-ICP-MS

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1 INTRODUCTION

While the importance of Zn as an essential but potentially toxic nutrient has been known for many years, it is only recently, since the completion of the human genome project, that we have come to appreciate just how many genes exhibit putative Zn binding motifs.[1] Conservative estimates indicate perhaps a thousand or more human genes may be zinc regulated[2] and, while we do not yet understand the functioning of many of these proteins, it is clear that Zn is an important cellular signal that can control many aspects of cellular functioning.

1.1 Metallothionein is Essential for Trace Metal Homeostasis

One protein that has been advocated to play a central role in intracellular metal metabolism and homeostasis in mammals and other organisms is metallothionein (MT).[3] Metallothioneins (MT's) represent a super gene family of highly conserved proteins that have been implicated in the redox mediated distribution of Zn in animal cells.[4] Four major isoforms of the protein have been identified in humans that are differentially expressed in various tissues.[5] Two of the better-characterized isoforms, termed MT-1 and MT-2, typify the primary structure of the gene family in having a high cysteine content (30%) and no aromatic residues. It is the uniquely high composition of cysteine that accounts for the redox sensitivity of these proteins and their ability to co-ordinate seven Zn or Cd atoms.[6] High resolution X-ray crystallographic studies have shown the protein to have a dumb-bell shape exhibiting 2 metal binding domains.[7] The N-terminal β-domains of each isoform contain nine cysteines coordinating 3 zinc atoms while the C-terminal α-domains are comprised of 11 cysteines, coordinating 4 zinc atoms. The metals are bound tetrahedrally within these domains via thiolate bonds that either form bridges between adjacent metal atoms or act as terminal ligands for the metal. Although these metal-thiol clusters are thermodynamically stable ($K_{D\ Zn}$ =1.2 x 10^{-13} M at pH 7.0),[8] studies have shown that the metal atoms within the cluster are relatively labile, particularly in the β-domain, permitting both intramolecular and intermolecular transfer of Zn within and between given MT isoforms.[9] This apparent lability, which can be increased by the presence of cellular oxidants, also accounts for the ability of the protein

to donate Zn to the apo form of various metalloenzymes having a lower affinity for the metal.[10, 11]

1.2 Considerations for Quantifying Transfer of Zn Between Metal-binding Ligands

The recent realization of the importance of Zn in life processes has underscored the need for not only a better understanding of Zn homeostasis and the role of various MT isoforms in this process, but also the development of new techniques to quantitatively monitor the movement of the metal from different sources and sinks in the cell. To date, three different approaches have been used to study the ability of MT to donate Zn, namely radiometric analysis, enzymatic reporter assays and electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS).[10, 12-14] Collectively these procedures can provide quantitative and kinetic information on the role of MT in the transfer process providing that extreme care and appropriate controls are undertaken to account for contaminating sources of metal, including adventitious metals associated with other ligands in the reaction. However, perhaps the best approach for unequivocally identifying and quantifying the MT-derived Zn is to label the donor protein with an isotope that can be distinguished from other, extraneous sources of metal. This has been accomplished in the past by the use of ^{65}Zn-radiolabeled MT.[11, 15] While gamma spectroscopy provides a highly precise and sensitive technique for metal quantification, accurate estimations of metal transfer require that the specific activity of the radiolabel on the various proteins be known before and after incubation. Radio and stable isotopic stoichiometry can be determined by using radiometric analysis in conjunction with a technique for total metal analysis, such as graphite furnace atomic absorption spectrophotometry (GFAAS) or inductively coupled plasma-optical emission spectroscopy/mass spectroscopy (ICP-OES/MS). Following incubation, exclusive MT to apo-enzyme Zn exchange can be confirmed by demonstrating identical specific activities for the recipient apo-protein after incubation as the MT prior to the reaction. These analyses require corrections to be made for counting efficiency and geometry and spectroscopic interferences, which can be time consuming and laborious. However, the biggest limitation in using ^{65}Zn as a tracer in this context is that only one donor protein can be studied at a time. This negates the possibility of studying interactions involving multiple donor and recipient proteins and, for this reason, very little is know about the functional differences of the various MT isoforms in Zn homeostasis within the cell. Thus, although the two major isoforms, MT-1 and MT-2, are co-expressed in the same tissues at the same time by the same inducers,[16-19] not much is known about their ability to exchange metals and act either as a common or independent Zn reservoir for metalloenzyme activation/inactivation.

1.3 Application of ICP-MS for Monitoring Zn Transfer Between Proteins

The following article describes a novel ICP-MS procedure that has the potential for quantifying Zn transfer between four different donor proteins while simultaneously allowing accurate assessments of contributions from contaminating sources to be taken into account. To unequivocally differentiate between the sources and sinks of metals in the transfer process, the procedure requires that the different donor proteins be differentially labeled with specific stable isotopes of Zn prior to incubation together. Following interaction, the proteins are separated and analyzed by directly coupled high performance liquid chromatography inductively coupled plasma mass spectrometry

(HPLC-ICP-MS). Metals on the resolved protein species are quantified by post column flow injection analysis (FIA) of analyte standards of known isotopic abundance and concentration. By using multiple recipient and donor proteins having different stable isotopic signatures, this technique has the potential for empirically studying and modeling the kinetic and thermodynamic aspects of Zn transfer between a number of competing ligands in relatively complex samples comparable to the cytoplasmic milieu. This is an important step to understanding the regulatory role of this metal in protein functioning and cellular metabolism.

To demonstrate the potential of this analytical method for evaluating new hypothesis about the mechanisms of metal exchange between metal-requiring proteins we have undertaken a number of clearly defined experiments using well characterized metalloprotein mixtures under non-physiological conditions. In the current study the procedure has been used to better understand the function and specific transfer properties of the two major MT isoforms (MT-1 and MT-2) and their ability to act as independent vectors in Zn transfer. More specifically, this paper describes the use of this procedure to study and model the time dependent intermolecular transfer of Zn between the two major MT isoforms, the relative transfer of Zn from the two isoforms to metal-depleted Alkaline Phosphatase (apo-AP), and the effect of apo-AP on the intermolecular transfer between the isoforms. To the best of our knowledge this is the first attempt to directly monitor inter-protein exchange of a metal by coupled HPLC-ICP-MS using two monoisotopically labeled proteins.

2 MATERIALS AND METHODS

2.1 Reagents and Materials

Rabbit liver metallothionein 1 and 2, bovine intestinal mucosa alkaline phosphatase Type VII-S, p-nitrophenyl phosphate (p-NPP), reduced glutathione (GSH), dithiothreitol (DTT), pyridine-2,6-dicarboxylic acid (PDC), 5',5'-dithiobis(2-nitrobenzoic acid) (DTNB: Ellman's reagent), acetonitrile, trifluoroacetic acid (TFA), Trizma® hydrochloride and base (Ultra-Pure) were purchased either from Sigma Chemicals or from Aldrich. Stable isotopic ^{67}Zn and ^{70}Zn were purchased from Oak Ridge National Laboratories. For the ^{67}Zn, the isotopic abundances of ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn were certified to be 1.113, 1.95, 94.6, 2.28 and 0.054 atomic % respectively with a precision of +/- 0.03% or better for all isotopes. For ^{70}Zn, double pass isotope was used with a certified isotopic ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn abundance composition of 0.13, 0.07, 0.02, 0.06 and 99.72 atomic % respectively with a precision of +/- 0.03% or better for all isotopes. Multi-element standards containing 10μg.mL^{-1} Zn, Cu, Cd, Ag and a single-element standard solution for Hg were obtained from SPC Science. Each element was certified at >99.99% purity. Solutions were prepared in acid washed, plastic ware using deionized water with a resistivity of <18MΩ.cm^{-1}. Adventitious metals in buffers and other preparative solutions were removed by filtration through a 2 x 40cm column packed with rejuvenated Chelex 100 beads (BioRad) at a rate of 1ml.min^{-1}. All solutions were checked for metal content by ICP-MS prior to use and rechelated if the Zn, Cu or Cd concentrations in the solution exceeded 0.05ng.mL^{-1}.

2.2 Coupled HPLC-ICP-MS Instrumentation and Instrumental Parameters

HPLC was performed at a flow rate of 1ml.min^{-1} using a "biocompatible" Beckman 126 programmable solvent delivery module equipped with a Beckman 168 diode array UV/VIS detector. PEEK® tubing was used to connect the various components in the system. A scavenger column (7.8mm ID x 75mm length), hand-packed with Chelex 100 resin (BioRad), was installed prior to a 100µL PEEK® sample injector loop to remove metals from the solvent stream. The system was flushed for 10 minutes with 10% HNO_3 to rejuvenate the scavenger column and passivate the system hardware. The system was subsequently flushed with deionized water and then the mobile phase before the attachment of the various size exclusion (SE) and weak anion exchange (AE) analytical columns.

Samples for directly coupled HPLC-ICP-MS were fractionated either isocratically by SE HPLC (600 x 7.8mm Biosep-SEC-S2000 column, Phenomenex) using a mobile phase of filtered, degassed 20mM Tris (pH 7.2) or by AE HPLC (75 x 7.8mm Showdex DEAE-825, column Phenomenex). A stepped gradient of 10mM Tris (pH 8.2) increasing to 10mM Tris in 500mM NH_4Cl (pH 8.2) over a 60-minute period was used to fractionate the proteins from the AE column. The UV/VIS absorbance of the eluting proteins was monitored between 190 and 600nm. Metals associated with the proteins in the solvent stream were analyzed using a PE-6100DRC ICP-MS directly coupled to the HPLC. A Rheodyne 7125 injector with a 200µL flow injection loop installed immediately prior to the ICP-MS was used to quantify the elemental composition of the resolved peaks and monitor analyte recovery.[20, 21] Sample aspiration into the ICP-MS was through a quartz Meinhard concentric nebulizer and a cyclonic spray chamber. The instrument was tuned daily using a 10ng.mL^{-1} solution of Li, Mg, Ce, Co, In, Ba and U to minimize oxide adduct formation and doubly charged species without unduly compromising sensitivity. Count rates were typically better than 600 x 10^3 cps for ^{115}In with a RSD of <0.5%. The Ce:CeO ratio was typically <0.02 under these operating conditions. Analysis and integration of the isotopic masses on the various peaks was performed using the Perkin Elmer Chromelink™ chromatographic application within Totalchrome™ software.

2.3 Preparation of Apo-Metallothionein (Thionein)

Thionein 1 and 2 were prepared by reverse phase HPLC using a modification of the method previously described by Vasak.[22] In brief, approximately 0.1mg of lyophilized Cd_7 or Zn_7MT-1 or 2 was dissolved in 100µL of a 0.1% TFA in 15% acetonitrile (pH 2.0). The sample was then further acidified by the addition of 10µL of 100% (TFA). Following 10 minutes of incubation, the released free metal was separated from the thionein by reverse phase chromatography. For chromatography, 100 µL of the crude acidified sample (0.1667mM MT) was applied to a Jupiter™ C_4 RP-300 column (5µ, 4.6 x 250mm; Phenomenex) and eluted at a flow rate 0.5ml/min using a stepped gradient increasing from 75% Buffer A (0.1% TFA, pH 2.0) to 100% Buffer B (0.1% TFA, pH 2.0 in 60% acetonitrile) over 60 minutes. The eluent was monitored continuously using a Beckman 168 photodiode array detector set to simultaneous record absorbances at 220nm (peptide bond), 254nm (Cd-thiolate bond) and 280nm (aromatic residues). The success of the procedure was monitored by the disappearance of the 254nm signature for the metal thiolate cluster coincident with the appearance of a peak showing strong absorbance at 220nm but not 280nm. Fractions conforming to these spectral criteria were collected by

hand and the presence of free thiols was quantified spectroscopically at 412nm by the reductive cleavage of DTNB. A standard curve of GSH was used to quantify the thiol content of the thionein assuming a thiol stoichiometry of 21:1 between thionein and GSH. The thiol content was normalized to the thionein protein concentration which was determined spectroscopically at 220nm in 0.1M HCl using a extinction coefficient of 48,200 $M^{-1}cm^{-1}$.[23] Thiol:apo-MT ratios of between 18-22 were typically obtained from the fractions as reported by others.[22]

Removal of the metals from the MT isoforms was confirmed, both by conventional ICP-MS analysis of a known concentration of thionein and by directly coupled AE-HPLC-ICP-MS (not shown). The latter procedure also provided an estimate of the potential for metal sequestration from the buffers, column and system hardware during chromatography.

2.4 Reconstitution of Thionein with ^{67}Zn and ^{70}Zn

Individual thionein fractions to be used for reconstitution were immediately frozen at -20°C after collection and quantification of free thiols. Thionein is relatively unstable at this temperature, even under anaerobic conditions, and reconstitution was typically performed within 1 hour of collection to prevent oxidation and loss of free thiols. All solutions were degassed by purging with He gas for 1 hour before use. Reconstitution with ^{67}Zn and ^{70}Zn was accomplished using an established procedure.[22] In brief, an 8 molar excess of 7 x ^{67}Zn or ^{70}Zn equivalents was added to the acidified thionein -1 or -2 and the mixture was slowly titrated over a 5-minute period with 0.5M Tris (pH 8.6) to a pH of 7.4. The sample was purged and agitated with helium gas during the titration process, which was monitored using a glass micro-pH electrode. Upon completion, an aliquot of 10mM dithiothreitol (DTT) in 20mM Tris (pH 7.4) was added to the sample to provide a final concentration of DTT of 0.1mM. Residual free $^{67}Zn/^{70}Zn$ in the reaction mixture was removed from the reconstituted protein by centrifugation to dryness under argon at 13,000g using a Microcon® (Amicon Bioseparations) centrifugal filter device with a molecular weight cut-off of 3,000Da. The sample was then rinsed with 4 x 300µL washes of 0.1mM DTT prepared in chelated 20mM Tris (pH 8.2) using the centrifugal device. The efficacy of the washing procedure in removing free Zn was determined by analyzing the various individual rinse solutions for $^{67}Zn/^{70}Zn$ by ICP-MS and was typically complete after the 4th rinse (not shown). The washed protein was then brought up to total volume of 50µL using the rinse buffer, and stored under argon at 4°C or, for longer periods, at –20°C. DTT in the ^{67}ZnMT-1 and ^{70}ZnMT-2 samples was removed prior to experimentation by rinsing further with 4 x 300µL washes of chelated 20mM Tris (pH 8.2 for AE-HPLC; pH 7.2 for SE-HPLC) as described above. The ^{67}ZnMT-1 and ^{70}ZnMT-2 were then brought to a total volume of 250µL with chelated 20mM Tris (pH 8.2) and quantified for metal content and isotopic purity by ICP-MS. DTNB analyses showed minimal free thiols in the sample indicating full reconstitution of the protein and the elimination of residual DTT in the buffer. The final concentration of each isoform was calculated from the metal content of the protein assuming a stoichiometry of 7g atoms Zn and Cd. $mole^{-1}$ and 11g atoms.$mole^{-1}$ for Cu. The MT isoforms were stored under Ar at 4°C and filtered through a chelated 0.2µm filter prior to use. A second batch of reconstituted MT isoforms with the reversed isotopic signatures (^{70}ZnMT-1 and ^{67}ZnMT-2) was also prepared as described as above.

2.5 Zinc Depleted Alkaline Phosphatase Preparation (Apo-AP)

Stabilizing ammonium sulfate in the stock AP solution was removed by centrifugation for 10 minutes at 13,000g at 4°C. The pellet was reconstituted back to the original volume in 1M Tris (pH 8.2) and the zinc in the protein was removed by chelating over a period of 3 days with 3 changes of an equal volume of Chelex 100 beads previously equilibrated with 1M Tris (pH 8.2). Removal of zinc was judged enzymatically by monitoring the reduction in the catalytic cleavage of p-NPP using 1µL of the AP-chelate sample mixed with 999µL of 1mM p-NPP suspended in 20mM Tris, pH 7.2. The reaction was allowed to proceed for three minutes at 37^{0}C at which time the absorbance of the sample was recorded with a dual beam spectrophotometer at 412nm. Upon completion of the chelation process, the apo-AP typically retained only about 1% of the original activity. The apo-AP concentration was determined using Coomassie Plus™ protein assay reagent (Pierce). The apo-protein preparation could be stored on Chelex beads for up to 3 days at 4°C, after which time there was progressive loss in reactivation upon the addition of exogenous Zn.

2.6 ZnMT-1 and ZnMT-2 Transfer Experiments

The two MT isoforms were incubated together in the presence and absence of apo-AP and/or free exogenous Zn. Initial reactions, monitoring the ability of each isoform to exchange Zn with the buffer, involved incubating 100 pmoles (0.5µM) of ^{70}ZnMT-1 or ^{67}ZnMT-2 with 700 pmoles of Zn in a total volume of 200µL(20 mM Tris pH 8.2) for one hour at 37°C. The added exogenous Zn exhibited normal geological isotopic abundance ratios and transfer from this source was monitored by the ^{64}Zn signal. The kinetics of inter-isoform exchange was monitored for periods of time ranging from 0 to 900 minutes by incubating mixtures of the two ^{67}ZnMT-1 and ^{70}ZnMT-2 under similar incubation conditions in the presence and absence of exogenously added free Zn. Metal transfer between the various proteins and from the free exogenous Zn was quantified by coupled AE-HPLC-ICP-MS using the ^{67}Zn, ^{70}Zn and ^{64}Zn (free Zn derived) signals respectively. Samples of 100µL were typically applied to the column and the degree of Zn exchange on each MT isoform was determined by quantifying the isotopic content of the integrated peak by FIA after correcting for isoform cross contamination. Zn transfer was expressed as % exchange (i.e. % Zn in MT isoform 1 from isoform 2) or atoms transferred (assuming a stoichiometry of 7g.atoms.mole^{-1}).

Reactions monitoring the donation of Zn from MT-1 and MT-2 to apo-AP involved incubating 50pmoles of ^{70}ZnMT-1 and ^{67}ZnMT-2 with 100 pmoles of apo-AP in a total volume of 100µL (0.5µM MT, 1µM apo-AP) for 1hour. These reactions were replicated with and without apo-AP in the presence of 700 pmoles of free, exogenous Zn to determine if transfer required a ligand exchange mechanism or could be mediated from free Zn in solution.

3 RESULTS

3.1 Isotopic and Elemental Purity of Isotopically Labeled ^{67}ZnMT-1 and ^{70}ZnMT-2

AE-HPLC-ICP-MS mass chromatograms of the reconstituted ^{67}ZnMT-1 and ^{70}ZnMT-2 show that the two isoforms can be resolved by their charge differences and elute at 4.7 and 15 minutes respectively (Figure 1, 0min). While there was evidence of traces of the other isoform in each preparation, the degree of cross contamination was highly consistent

between injections (5.65% ^{67}ZnMT-2 in ^{67}ZnMT-1, and 5.41% ^{70}ZnMT-1 in ^{70}ZnMT-2), which allowed for correction during subsequent calculations of metal transfer. Two small peaks for metals observed at 2.6 and 3.3 minutes in both the isoform preparations were due to anionic materials in the void and oxidation products of MT respectively. The increasing signal at m/z 67 after 17 minutes was due to the formation of $Cl^{35}O_2^{16}$ polyatomic interferences generated by the NH_4Cl gradient.

Quantitative analyses of the integrated peaks for the individual isoforms given in table 1 show efficient reconstitution with high elemental purity and negligible ^{64}Zn, Cd, Cu, Ag or Hg contamination from extraneous metals during preparation or chromatography. Similarly, isotopic analyses show that the isoforms are essentially mono-isotopic $^{67}Zn_7$MT-1 and $^{70}Zn_7$MT-2 with an isotopic composition comparable to that of the starting material used to reconstitute the apo-protein (table 1). Similar purity was observed for the ^{70}ZnMT-1 and ^{67}ZnMT-2 preparations (data not shown).

Table 1. *Relative atomic % abundances of various isotopic masses based upon the mean response from duplicate AE HPLC-ICP-MS analyses of 100µL injections of a 0.5 µM solution (50 pmoles) of $^{70}Zn_7$MT-1 and $^{67}Zn_7$MT-2.*

Element/Mass	***Relative % Atomic abundance***	
	$^{67}Zn_7$MT-1	***$^{70}Zn_7$MT-2***
Cd	0.27	0.54
^{64}Zn	1.47	1.47
^{66}Zn	1.63	0.83
^{67}Zn	94.59	0.07
^{68}Zn	1.27	0.56
^{70}Zn	0.02	97.13
Cu	0.74	ND
Ag	ND	ND
Hg	ND	ND

3.2 Zn Exchange Between ^{67}ZnMT-1 and ^{70}ZnMT-2

The time dependent transfer of Zn between the two MT isoforms was followed discontinuously by AE-HPLC-ICP-MS by sub-sampling the mixture periodically over the incubation period (Figure 1). Rapid reciprocal transfer of Zn between the two isoforms was observed with equilibration being essentially complete by 900 minutes (Figure 2). Metal exchange occurred exclusively between the two isoforms and there was no evidence of exchange with extraneous ^{64}Zn, ^{65}Cu or ^{114}Cd in the buffer during the incubation period or from the HPLC column during chromatography (Figure 3).

A series of reactions were conducted to test the effects of free Zn in solution on the metal exchange between the two isoforms. The incubation conditions were maintained as before with the exception that the isotopic signatures of the two MT isoforms were reversed (i.e. ^{70}ZnMT-1 and ^{67}ZnMT-2, Figures 4a,b). Initial reactions were conducted to determine the ability of each of the individual isoforms to exchange metal with free Zn in solution. For these reactions, 700 pmoles of free Zn (natural isotopic abundance) was added to 100 pmoles of each of the MT isoforms (equivalent to a 1:1 ratio of MT-derived to free Zn; 20mM Tris, pH 8.2) (Figures 5a,b). Analyses, based on the ^{64}Zn content of the

isoform corrected for isotopic abundance after 1hour of incubation at 37^{o}C, showed that 24% of the Zn in MT-1 and 21% of the Zn in MT-2 originated from the free Zn pool. This equated to approximately 1.7 of the 7 atoms having originated from the medium (figure 7). This exchange was comparable to that observed between the two MT isoforms in the absence of free Zn under otherwise identical conditions, which indicated that 32% and 26% of the metal associated with MT-2 and MT-1 respectively was derived from the other isoform (Figures 6a, 7). The addition of a 7 fold molar excess of free Zn to a mixture of MT-1 and MT-2 (i.e. 100 pmoles MT-1, 100 pmoles MT-2, 700 pmoles of free Zn in 200 µL Tris, pH 8.2) caused a slight increase in the mass of Zn transfer from MT-1 to MT-2 (Figure 5c). Transfer from MT-2 to MT-1 was only slightly reduced under these conditions (c.f. Figures 5c, 6a, 7), as was the transfer of free Zn from the buffer relative to incubations having only one isoform (c.f. Figures 5a,b,c, 7).

3.3 Zn Transfer and Activation of Apo-AP with ^{67}ZnMT-1 and ^{70}ZnMT-2

Although exhaustive chelation of AP with Chelex 100 caused a significant reduction in enzymatic activity, AE-HPLC-ICP-MS chromatograms of apo-AP showed the presence of trace quantities of residual ^{64}Zn eluting at 16.5 minutes in association with an unknown contaminant and at 20.5 minutes with the Zn-depleted enzyme (Figure 4c). Incubations involving the simultaneous addition of 100 pmoles of apo-protein with 50 pmoles of MT-1 and MT-2 showed the preferential transfer of Zn from MT-1 to apo-AP over MT-2 after one hour, with approximately twice as much metal being donated from ^{67}ZnMT-1 than ^{70}ZnMT-2 (data not shown). Quantitatively this transfer equated to a 4.3 fold increase in the residual Zn associated with the apo-AP which caused a commensurate 4.9 fold increase in the catalytic activity in the enzyme (table 2). Interestingly, the addition of apo-AP to the reaction caused a dramatic 3-fold reduction in Zn exchange between the two isoforms over the 60-minute incubation period (data not shown). No Zn transfer to the contaminant in the preparation was observed during chromatography and this peak showed no enzymatic activity.

Table 2. *Enzymatic Activation of apo-AP following Zn transfer from MT-1 and 2.*

Treatment[a]	***Zn on AP (pmoles)***	***Activity (au.min^{-1}.nmole^{-1})***[b]
Apo-AP (control)	15.35	5.5 +/- 0.53
Apo-AP + ^{67}ZnMT-1 + ^{70}ZnMT-2	66.7	27.21+/- 0.72

[a] Conditions: 50pmoles MT-1 and MT-2, 100pmoles apo-AP, 1 hour incubation, 20 mM Tris pH 8.2, reaction volume 100µL.

[b] au = absorbance units (Mean +/- Standard error of mean, n = 3.)

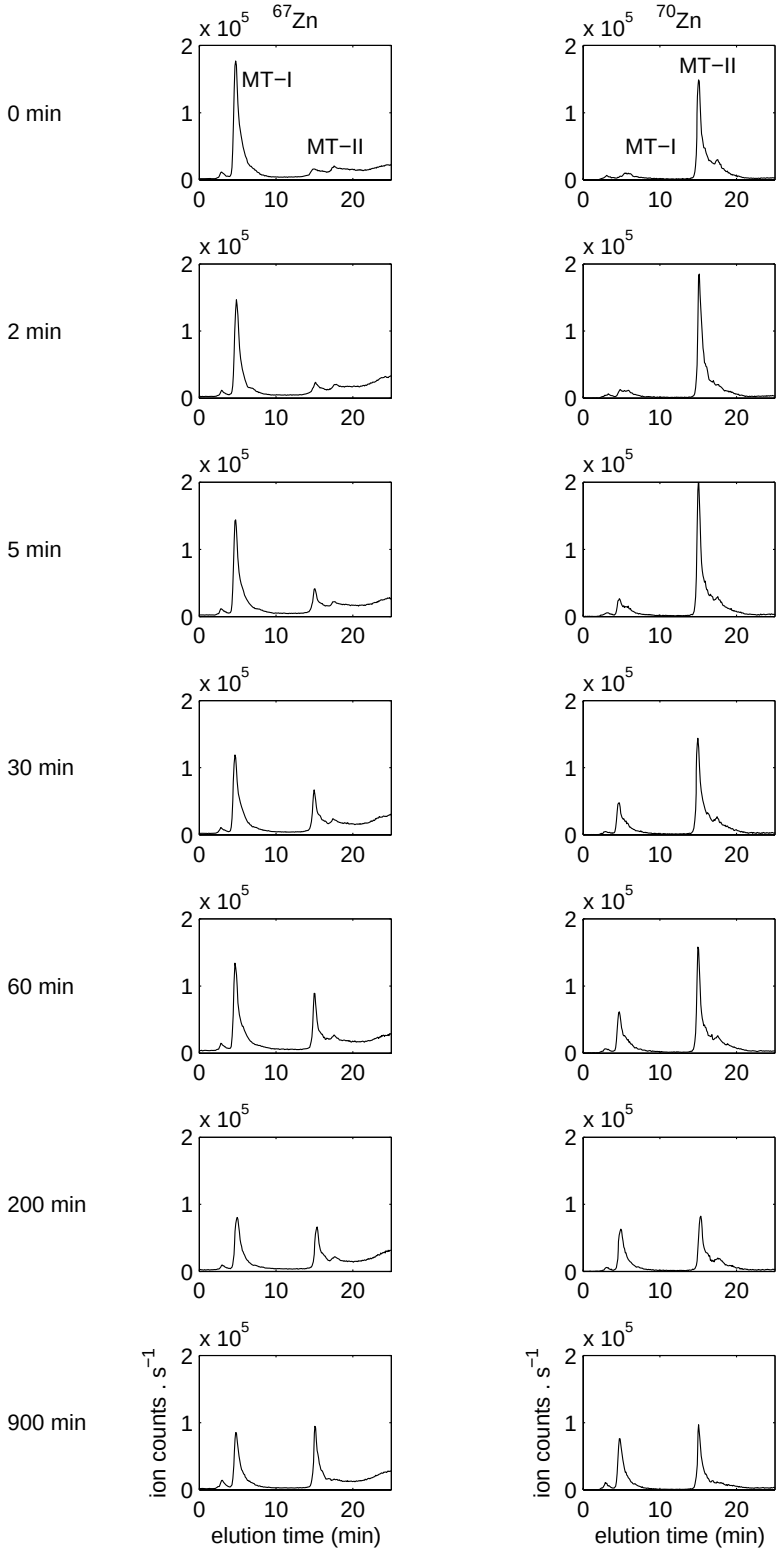

Figure 1 *AE-HPLC-ICP-MS elution profiles showing the exchange of ^{67}Zn and ^{70}Zn between monoisotopically labeled ^{67}ZnMT-1 and ^{70}ZnMT-2 incubated together at 37^{o}C for periods of time ranging from 0 to 900 minutes. 100µl injections of 0.5µM ^{67}ZnMT-1 and ^{70}ZnMT-2 were applied to the column (20 mM Tris pH 8.2). The 0 minute elution profiles correspond to measurements of ^{67}ZnMT-1 and ^{70}ZnMT-2 prior to mixing.*

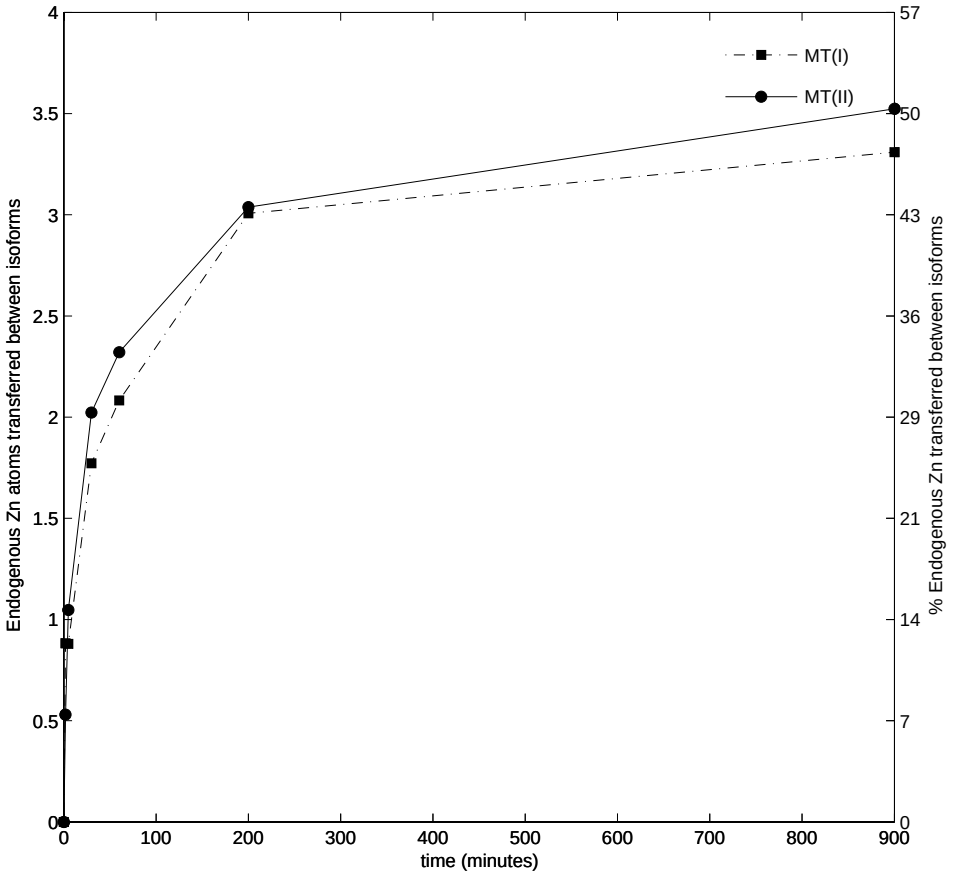

Figure 2 *Quantification of Zn transferred between MT-1 and to MT-2 over a 900-minute incubation period. Values are expressed both as atoms exchanged and the percentage of Zn transferred as calculated from the co-elution of the Zn isotopes with the two MT isoforms.*

This experiment was repeated using 66.4 pmoles of apo-AP and 100 pmoles of each MT isoform in the presence and absence of 700 pmoles of naturally isotopic free Zn, the isoforms having reversed isotopic signatures in these incubations (i.e. ^{70}ZnMT-1 and ^{67}ZnMT-2). Calculations comparing the distribution and sources of Zn associated with the two MT isoforms and the apo-AP after 1 hour of incubation again showed that MT-1 donated approximately twice as much Zn to the apo-protein as MT-2 (Figures 6c,6d). Similarly, intermolecular exchange of Zn between the isoforms was quenched in the presence of apo-AP (c.f. Figures 6a and 6c). Thus, the exchange from MT-1 to MT-2 was reduced 3 fold while transfer from MT-2 to MT-1 was essentially abolished (Figure 7). Saturation of the apo-AP by the addition of 700 pmoles of free Zn to the solution caused the resumption of inter-isoform metal exchange (Figure 6e). This rapid sequestration of ^{64}Zn by the apo-enzyme also simultaneously reduced the amount of metal being transferred to the protein from the two MT isoforms (c.f. Figures 6d and 6f, Figure 7), although MT-1 still donated approximately twice as much metal as MT-2 (Figure 6f, 7).

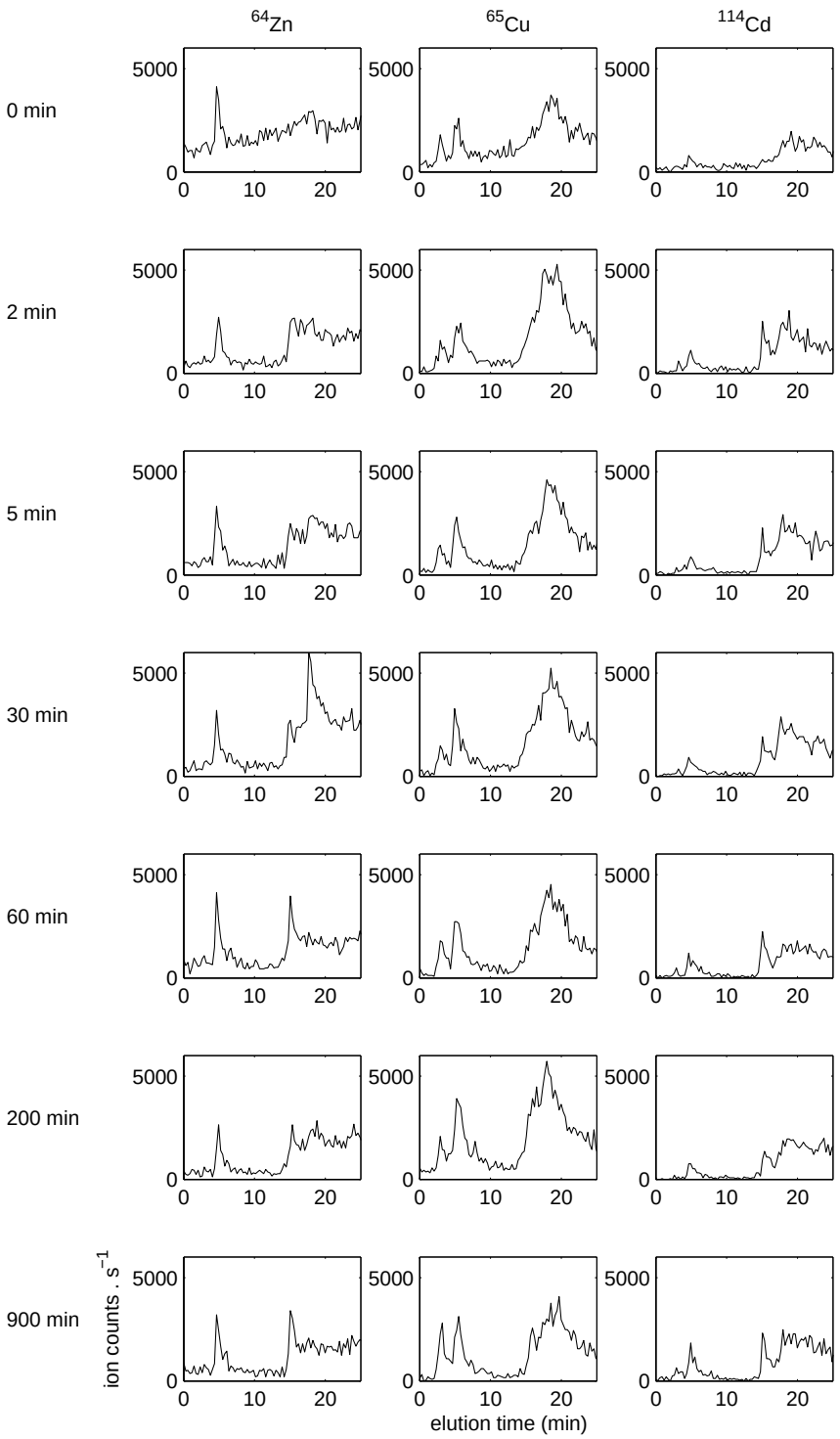

Figure 3 *AE-HPLC-ICP-MS elution profiles of ^{64}Zn, ^{65}Cu, and ^{114}Cd in mixtures of monoisotopically labeled ^{67}ZnMT-1 and ^{70}ZnMT-2 incubated at 37^{o}C for periods of time ranging from 0 to 900 minutes.*

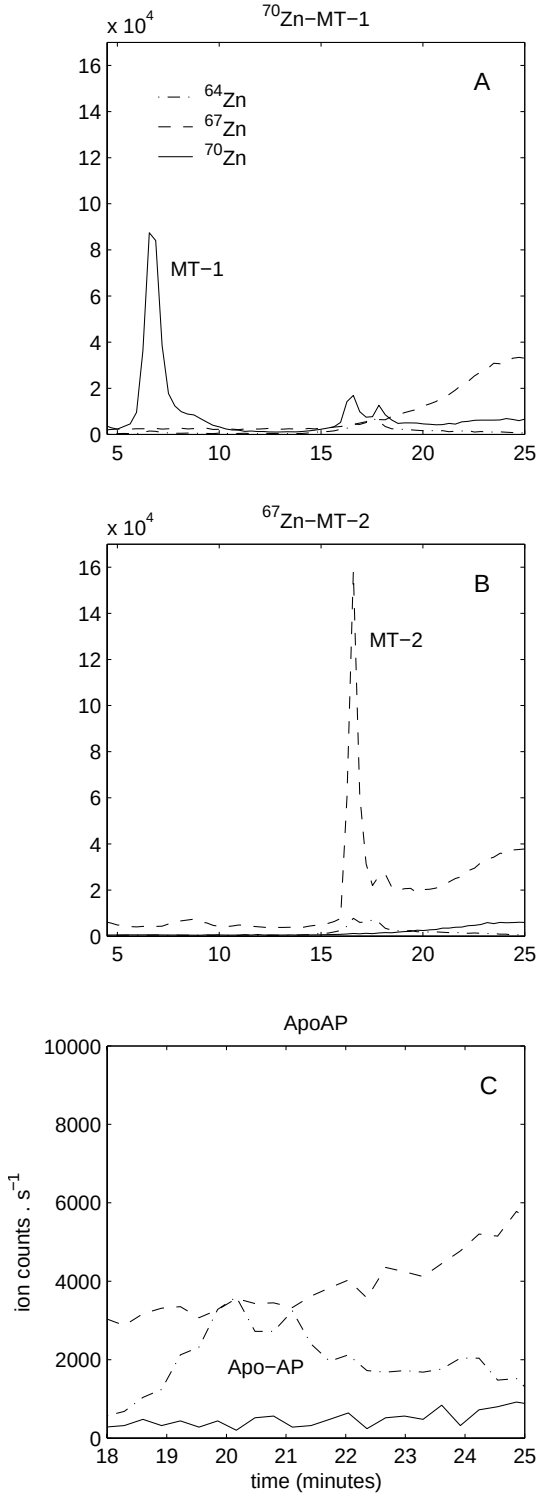

Figure 4 *AE-HPLC-ICP-MS elution profiles of ^{64}Zn, ^{67}Zn, and ^{70}Zn in monoisotopically labeled (A) ^{70}ZnMT-1 and (B) ^{67}ZnMT-2 and (C) apo-AP. Although nascent activity in the apo-AP preparation was only 1%, clearly not all the Zn could be chelated in the apoprotein preparation.*

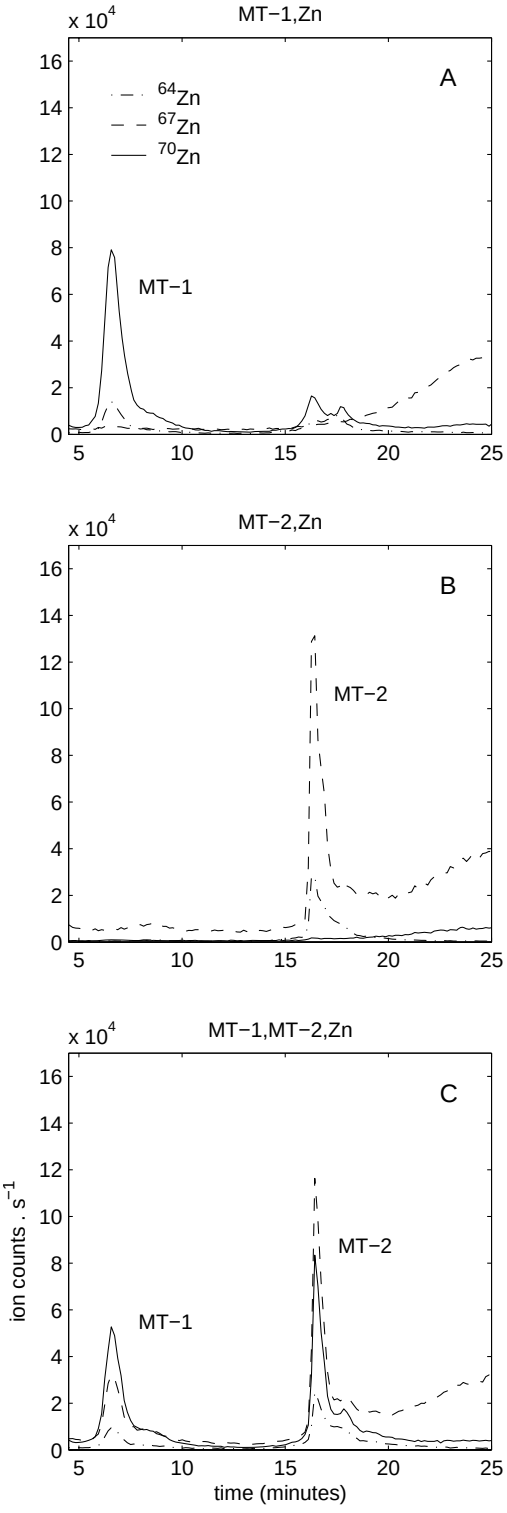

Figure 5 *AE-HPLC-ICP-MS elution profiles of ^{64}Zn, ^{67}Zn, and ^{70}Zn in monoisotopically labeled (A) $^{70}ZnMT$-1 and (B) $^{67}ZnMT$-2 and (C) a 1:1 mixture of the two isoforms incubated at 37^{o}C for 1 hour with 700 pmoles of free Zn. The ^{64}Zn signal is used to quantify the total Zn load on the isoforms that originated from the buffer rather than inter-isoform transfer.*

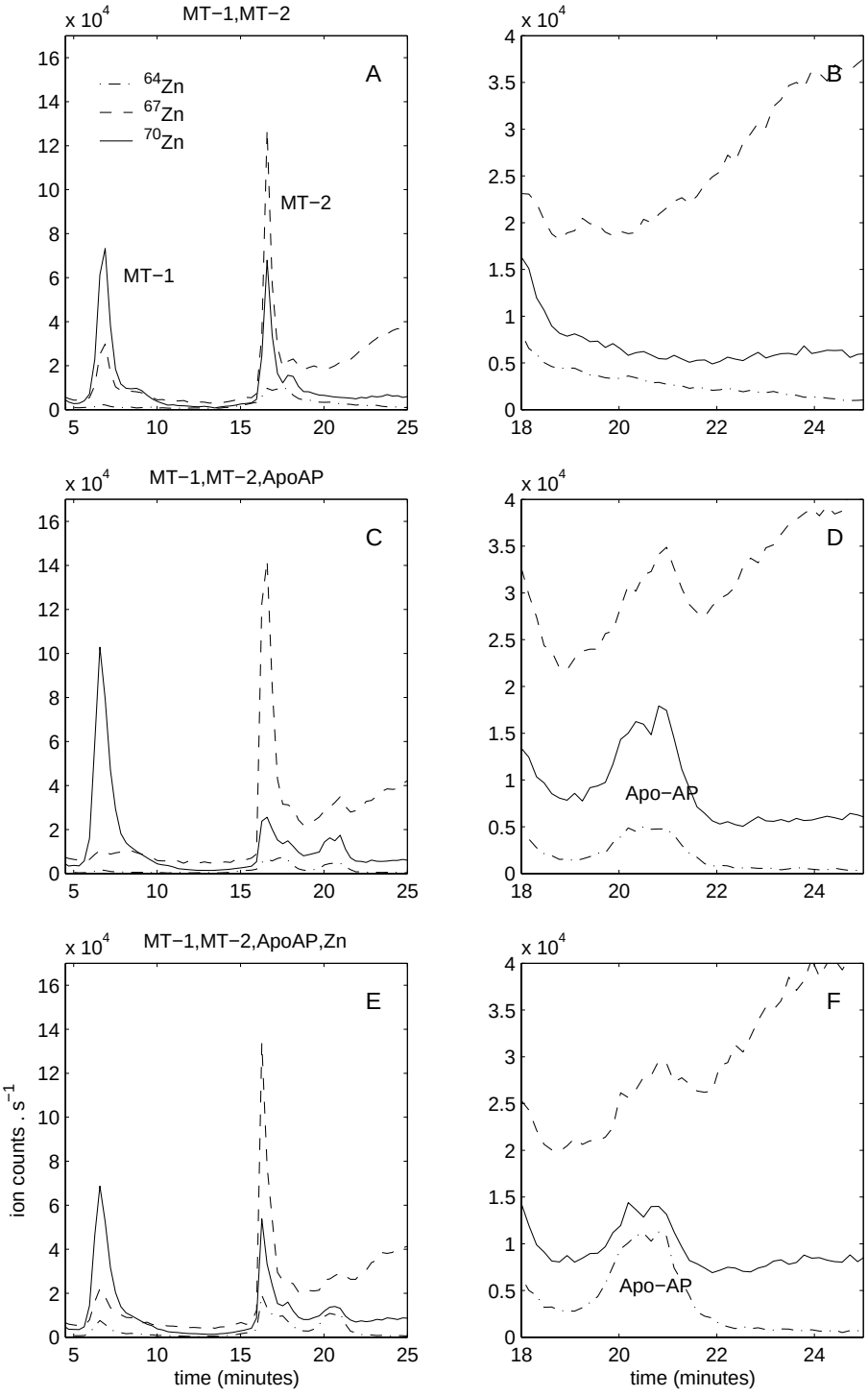

Figure 6 *AE-HPLC-ICP-MS elution profiles of ^{64}Zn, ^{67}Zn, and ^{70}Zn in 1:1 mixtures of ^{70}ZnMT-1 and ^{67}ZnMT-2 incubated by themselves (A), with apo-AP (C) and with apo-AP and 700 pmoles additional Zn (E) for one hour under the described conditions. The panels (B), (D), and (F) rescale the chromatograms of (A), (C), and (E), respectively, to emphasize the apo-AP peak.*

4 CONCLUSIONS

Recent studies on MT have implicated these proteins in both metal detoxification and homeostasis, although the roles of the individual isoforms in each process remain unclear.[3, 24] While it has been acknowledged that the co-expression of the two major isoforms implies that they may have important functional differences, we have not had the technology to monitor the role of each isoform in metal metabolism independently when mixed together in a reaction. In the present study we have developed a protocol, using monoisotopically labeled $^{67}Zn_7$MT-1 and $^{70}Zn_7$MT-2 and coupled HPLC-ICP-MS, to directly quantify the intermolecular transfer of Zn between the two protein isoforms as well as to study their ability to differentially donate Zn to the apo metalloenzyme apo-AP. Since the natural isotopic abundances of ^{67}Zn and ^{70}Zn are 4.1% and 0.62% respectively, the procedure can readily distinguish between metal transferred from each isoform, while isotopic analyses considering the ^{64}Zn content can be used to determine the Zn contributed from extraneous sources. Following incubation, the various participants in the reaction are separated by anion exchange HPLC and their isotopic content of Zn analyzed by ICP-MS and quantified by post column FIA using serial dilutions of a multi-element standard with known isotopic abundances. While the elemental detection limits (~ p-nM) and precision (~ 0.1-1%) of this technique are generally inferior to radiometric analysis, the procedure does have a number of distinct advantages over using a radiolabeled protein. Thus, only one analysis has to be conducted on each sample, the isotopic profiles are essentially correction and interference free and, perhaps most importantly, the technique has the ability to simultaneously quantify the movements of metal from four potential Zn donors. The technique has the added advantage that it can also simultaneously track the movements of other metals between the various participants in the reaction. In this way, the procedure has the ability to monitor for competitive and synergistic interactions between metals for cellular ligands, which is probably a key to understanding the homeostatic mechanisms used by cells to ensure that cellular processes are simultaneously protected from metal deficiencies and excesses.[25]

4.1 Zn Exchange Between ^{67}ZnMT-1 and ^{70}ZnMT-2

Our initial reactions focusing on the exchange of Zn between MT-1 and MT-2 demonstrate rapid and equivalent intermolecular transfer between the isoforms. These observations are in general accord with the works of others who have shown that, despite its high affinity for Zn, MT can exchange metal rapidly between isoforms[15] and with Zn in solution.[26] The mechanism of transfer of Zn between MT isoforms is unclear. Structurally the Zn in the domains is buried within the protein and, because each atom is tetrahedrally coordinated, it has been proposed that transfer must occur via a ligand exchange mechanism involving partitioning through transient inter-molecular metal-thiolate bonds.[27] Support for this proposition is provided by dialysis studies that have shown that Zn exchange cannot occur between isoforms when they are separated by a membrane permeable to free Zn but not to the protein.[28] Ligand-ligand exchange requires the formation of transient complexes and evidence for the formation of metallothionein dimers in solution has been reported by a number of workers. These dimers appear to form under two conditions. First, in the presence of excess Cd, Palumaa et al.,[29, 30] showed the formation of a S-Cd-S bridge between the β-domains of adjacent monomers that increased metal exchange between the bridged molecules forming the dimer. Second,

oxidative conditions can also cause dimerization. In this case, dimerization appears to be mediated via a disulfide bridge involving Cys-36 in the 4-metal cluster at the C-terminal α-domain.[31] Despite the loss of a coordinating thiol bridge within the domain, all 4 metals are retained in the cluster and there appears to be no increased metal lability in either domain.[31]

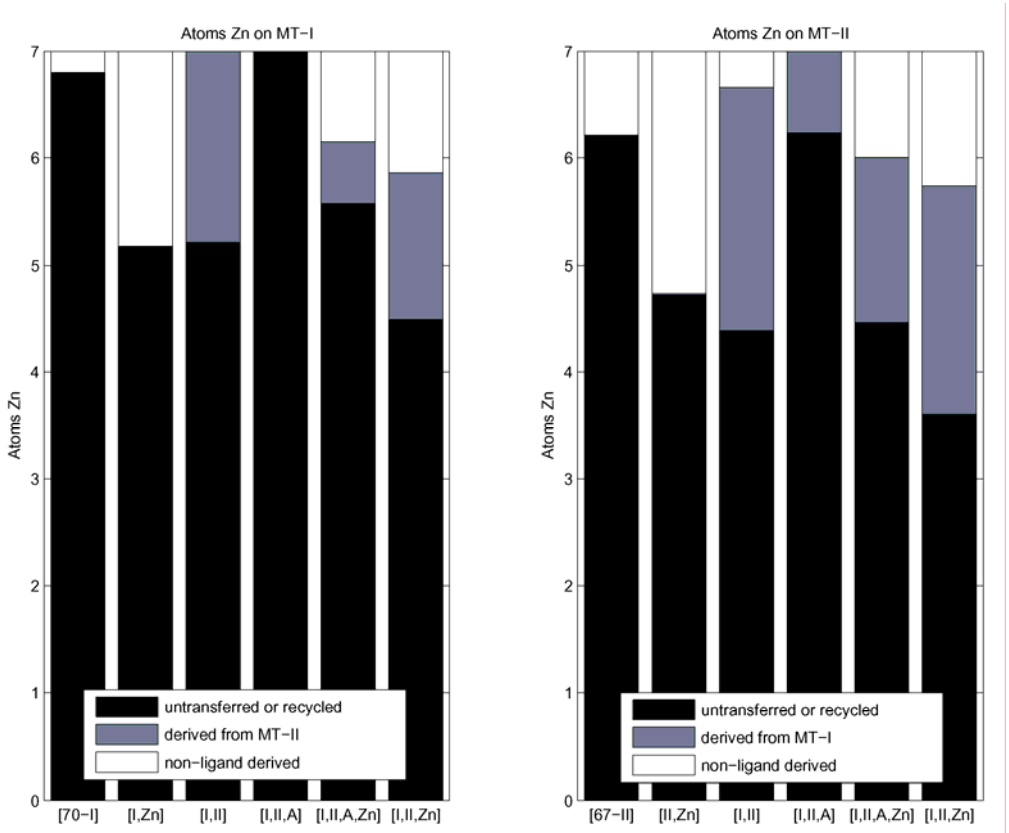

Figure 7 *Calculations showing the origin of each Zn atom bound to MT-1 (left) and MT-2 (right) under different experimental conditions (x ordinate). The codes [70-I] and [67-II] represent control measurements of the monoisotopically labelled isoforms. In other codes: I, II, A, and Zn represent MT-1, MT-2, apo-AP and 700 pmoles free Zn, respectively. Thus [I,II,A] would represent the incubation of MT-1, MT-2, and apo-AP for one hour under the previously described experimental conditions. Calculations are normalized to a theoretical saturation of 7 atoms of Zn for each isoform.*

Our data cannot directly differentiate between transfer through ligand exchange or a transfer process mediated primarily via the buffer. Indeed, the behavior of inter-isoform metal transfer shown in our study is complex and cannot be readily rationalized by either transfer processes. The ability of both MT-1 and MT-2 to sequester 2 atoms of exogenous zinc derived from the buffer clearly establishes that direct exchange between the MT and free Zn occurs. This would appear to favor exchange mechanisms via buffer transfer, presumably via the solvent accessible atom that has been identified in the labile β-domain of the molecule.[31]

Continued exchange with Zn in the buffer together with concomitant intramolecular self-exchange between the two domains would eventually give rise to

equilibration and isoforms with comparable isotopic signatures. Under the experimental conditions used in the current study this appears to occur after approximately 900 minutes.

Inter-isoform Zn exchange via the buffer would also help explain the remarkable inhibition exchange caused by the addition of apo-AP observed in these studies. Thus, by sequestering the free ^{67}Zn and ^{70}Zn in the buffer as it dissociates from the MT isoforms, the apo-protein could act as a "kinetic trap" that intercepts the metal rendering it unavailable for inter-isoform transfer. Accordingly, the addition of 700 pmoles of free Zn to the solution rapidly saturates the apo-AP which, because it exchanges its bound metal relatively slowly with the free Zn in solution, restores inter-isoform Zn transfer to values comparable to those noted in the absence of apo-AP. Therefore, at first glance, it would appear that inter-isoform metal exchange occurs via metal release and uptake from the buffer. If this assumption were correct, then the exchange of Zn between isoforms in the present study should be slower than the rate of exchange with free Zn in solution and should also be severely inhibited by the addition of an excess of exogenous free Zn to the reaction. However, under stoichiometric conditions (700 pmoles of ^{70}Zn, 700pMoles ^{67}Zn and 700 pmoles ^{64}Zn exogenous), our data show the kinetics of exchange between isoforms to be comparable to the rate of exchange with free Zn and unaffected by exogenous metal. These kinetics are clearly inconsistent with inter-isoform exchange occurring via free metal in solution and it is necessary to invoke a model involving metal transfer through a ligand-ligand exchange process to reconcile these apparently conflicting interpretations.

4.2 Proposed Mechanism of Zn Exchange Between ^{67}ZnMT-1 and ^{70}ZnMT-2

We speculate that inter-isoform exchange between MT-1 and MT-2 under the present conditions occurs almost exclusively via ligand exchange between Zn-bridged dimers in a manner similar to that proposed by Zangger and Armitage for Cd.[31] For cadmium, these non-oxidative bridges form due to the presence of excess of Cd^{2+} in the buffer and, given the high concentrations of MT isoforms in our incubations (0.5μM), the conditions for dimerization are probably met in the majority of our reactions irrespective of whether exogenous Zn is added. Depending upon the specific reaction conditions, the bridging atom would be provided either by ^{70}Zn and/or ^{67}Zn released from MT or from unligated ^{64}Zn added to the reaction mixtures. By promoting homo- and hetero-dimer formation, these bridges presumably facilitate both intra and inter-isoform metal exchange. Because exchange is primarily between the atoms already located in the β-domains of the individual molecules forming the dimer and not between the bridging atom and the domain, an exogenously added excess of ^{64}Zn does not inhibit the inter-isoform exchange of ^{70}Zn and ^{67}Zn, as demonstrated in our reactions. Significantly, the one situation where the conditions for dimerization are not met in the current study is when apo-AP is added to the isoform mixture. Here, the apo-enzyme scavenges the free ^{70}Zn and ^{67}Zn from solution preventing S-Zn-S bridge formation, causing dissociation of the existing dimers and thereby preventing protein-protein exchange. The kinetics of dimer dissociation involving Cd bridges has been shown to proceed slowly upon the addition of the chelating agents 2β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA),[29] taking hours to complete. Our data suggest that the transient Zn bridges are either weaker than those formed with Cd, or that they form relatively slowly compared to the binding kinetics of apo-AP to the unligated Zn, since samples of MT-1, MT-2 and apo-AP mixed simultaneously upon incubation show negligible inter-isoform transfer (Figure 6c).

Naturally, the addition of exogenous ^{64}Zn to the reaction saturates the apo-AP, causing the reestablishment of the bridges and inter-isoform transfer as shown in Figure 6e.

A key to understanding whether this inter-isoform exchange has any cellular significance *in vivo* is the threshold of free Zn^{2+} necessary to initiate the process. Cellular free Zn^{2+} values of between 5 x 10^{-10} and 2.4 x 10^{-11} have been quoted in the literature which approximate closely to the stability constants for many eukaryotic zinc containing proteins, which have –log K_D values in the range of 10-12.[32] It is therefore generally accepted that the concentration of unligated Zn^{2+} in the cell is between 10^{-9}M and 10^{-12}M,[33-35] being bounded at the upper end of the range by metal induced enzyme inactivation and at the lower end by cellular deficiencies giving rise to apo-enzyme formation. Some of our preliminary data on the conditions necessary for isoform transfer have shown that the threshold for exchange is very sensitive and can be established by titration experiments using variable amounts of apo-AP and free Zn (not shown). However, more detailed experiments are necessary to establish whether these *in vitro* reactions conducted under non-physiological concentrations can be extended to more relevant cellular conditions. In the event that the threshold for MT dimerization and inter-isoform exchange falls within the physiological range of free Zn in the cell, then one could envisage a scenario where oscillations in the cellular concentrations of unligated Zn^{2+} could act to trigger either kinetic isolation or communication between the various pools of Zn associated with specific MT isoforms.

4.3 Modelling Zn Exchange Between ^{67}ZnMT-1 and ^{70}ZnMT-2

To evaluate the mechanism of metal exchange between the MT isoforms, we developed a multi-compartment mass-balance model that incorporated some of the known structural and thermodynamic parameters of MT. Figure 8 shows a schematic of the compartment model and associated kinetic parameters.

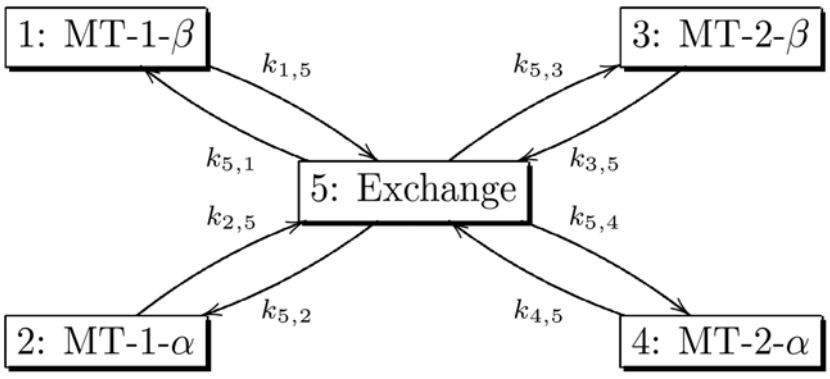

Figure 8 *Schematic of a multi-compartmental model of Zn exchange between MT isoforms and corresponding rate constants for exchange (See Table 1 for values). The α- and β- domains of MT-1 and MT-2 are represented as kinetically separate pools numbered 1-4 that exchange Zn via an abstract exchange pool (pool 5). The numbering of compartments and rate constants correspond to the model equations given in the text (1-3).*

It was assumed that the MT-α and MT-β domains of each isoform constitute independent and non-cooperative kinetic pools of metal and that all metal exchange occurs through a ligand exchange mechanism that requires an excess of free Zn in the buffer or between an MT molecule and free Zn. Efflux of metal from the domain pools was assumed to follow the law of mass action with rate constants estimated from published K_D values for the respective isolated MT domains.[36] A mathematical expression describing transfer of metal from the "exchange pool" to each domain required a statistical argument relating the probability of a binding event occurring as a function of the degree of metal saturation of the domain. The "exchange pool" is an abstract pool that encompasses both ligand-ligand exchange of metal and ligand-metal exchange with the physical buffer. For example, if MT-α is less than ¾ saturated with metal then, on average, the domain will be able to bind one additional Zn atom and virtually all collisions (either ligand-ligand or metal-ligand) will result in binding events. We assumed that metal-ligand and ligand-ligand collisions are near diffusion limited. Table 3 shows all kinetic parameters and their estimated values.

Table 3. *Rate constants for multi-compartment mass balance model of Zn transfer between monoisotopically labeled MT-1 and MT-2*

Rate Constant	Value	Explanation	Reference
k_a (Diffusion)	1e-3 $(pM \cdot s)^{-1}$	Approximate diffusion limited rate empirically adjusted to give best model fit.	N.A
$k_{1,5}$ $k_{3,5}$	5e-3 s^{-1}	Studies of isolated MT-1-β domain estimate a K_d = 5pM for Zn. Assuming diffusion limited binding of Zn with MT the rate constant is calculated as $K_d k_a$.	36
$k_{2,5}$ $k_{4,5}$	1.4e-4 s^{-1}	Studies of isolated MT-1-α domain reveal it to be less Zn-labile than the MT-1-β domain but with cooperative change in affinity upon the addition of Zn atoms. For lack of exact parameters, we assume that each Zn behaves identically and set the $K_d = 0.14\,pM$, which is approximately the average K_d for the entire MT molecule.	36
$k_{5,i}$		See equation (3) in text	

The above model can be written as a system of linear differential equations that describe the time-dependent changes of ^{67}Zn and ^{70}Zn. Let i = 1…5 denote the compartment in Figure 8, $k_{i,j}$ denote the rate of exchange between compartments i and j, and $C_i^n(t)$ denote the time dependent concentration of ^{n}Zn in compartment i. For influx and efflux of Zn isotope from the MT ligand-pools we have:

$$\begin{aligned}
\frac{\Delta \text{ isotope}}{\Delta t} &= \text{INFLUX - EFFLUX} \\
\frac{dC_i^{67}(t)}{dt} &= k_{5,i}C_5^{67}(t) - k_{i,5}C_i^{67}(t) \\
\frac{dC_i^{70}(t)}{dt} &= k_{5,i}C_5^{70}(t) - k_{i,5}C_i^{70}(t)
\end{aligned} \tag{1}$$

Compartment 5 defines the exchange between all pools so it is written as:

$$\begin{aligned}
\frac{\Delta \text{ isotope}}{\Delta t} &= \text{INFLUX - EFFLUX} \\
\frac{dC_5^{67}(t)}{dt} &= \sum_{i=1}^{4} k_{i,5}C_i^{67}(t) - \sum_{i=1}^{4} k_{5,i}C_5^{67}(t) \\
\frac{dC_5^{70}(t)}{dt} &= \sum_{i=1}^{4} k_{i,5}C_i^{70}(t) - \sum_{i=1}^{4} k_{5,i}C_5^{70}(t)
\end{aligned} \tag{2}$$

The kinetic parameter for efflux of metal from the exchange compartment varies according to the saturation of the corresponding MT domain pool. Let n be the number of atoms bound to an MT domain when fully saturated, $[\text{Zn}_i^{\max}]$ denote the maximum Zn capacity of the compartment, $[\text{L}_i]$ denote the concentration in pMoles of the MT domain corresponding to compartment i=1…4, then:

$$k_{5,i} = \begin{cases} k_a[\text{L}_i] & \text{if } \dfrac{C_i^{67}(t) + C_i^{70}(t)}{[\text{Zn}_i^{\max}]} < \dfrac{n-1}{n} \\ \left(1 - \dfrac{C_i^{67}(t) + C_i^{70}(t)}{[\text{Zn}_i^{\max}]}\right)k_a & \text{otherwise} \end{cases} \tag{3}$$

The solution for this system was solved using a standard 4$^{\text{th}}$ order Runga-Kutta algorithm in the MATLAB™ programming environment.

Although simplified by assuming identical metal-binding characteristics for MT-1 and MT-2, the model predicts a time-course of metal exchange between the two isoforms (Figure 9d) that agrees well with our experiments by accounting for the differing metal-binding properties of the MT-β (Figure 9a) and MT-α (Figure 9b) domains. While a compartmental model necessarily hides important mechanistic information behind its abstraction of flux between compartments, it nonetheless provides a convenient framework for rationalizing complex metal-ligand equilibria. By judiciously choosing rate constants for physical compartmental structures that are consistent with and conform

to prior biological knowledge, these models can have important predictive properties. In future work we intend to explore the use of such models for understanding the effects of redox state on the time dependent exchange of Zn between isoforms.

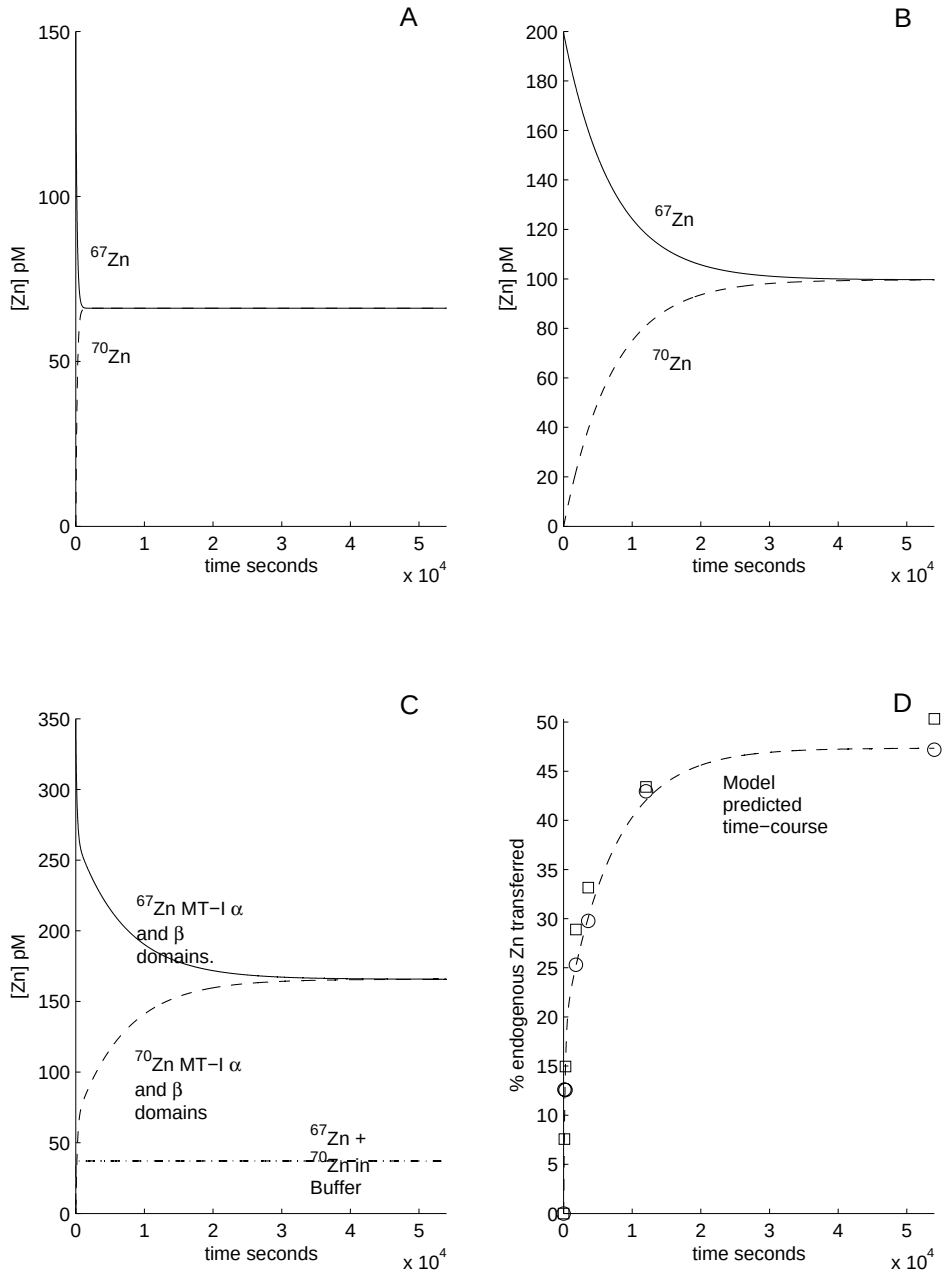

Figure 9 *Predicted time-course of turnover and exchange of Zn in the β-domain (A), α-domain (B), and whole MT molecule and the exchanging Zn in compartment 5 where free Zn can be loosely interpreted as present in the "buffer" solution (C) after solving the system of linear differential equations that describe the proposed mass balance model. The model does not differentiate the biophysical properties of the MT isoforms and thus only the time course for MT-1 is shown. Panel D shows the results of the simulation expressed as percentage endogenous Zn transferred along with the experimental data for MT isoforms 1 and 2.*

4.4 Zn Transfer and Activation of Apo-AP with ^{67}ZnMT-1 and ^{70}ZnMT-2

Although the thermodynamically unfavorable donation of Zn from MT (K_D MT = 1.4 x 10^{-13}) to apo-AP (K_D AP 1.67 x 10^{-12}, pH 8.6)[37] has been demonstrated in this and other studies,[38] the mechanism of this transfer is unclear. The release of Zn from MT for apo-metalloenzyme activation has been shown by others to be redox modulated by the GSH/GSSH couple, which may make the zinc in the thiolate clusters of MT more labile and accessible for donation. The redox sensitivity of MT in Zn release resides with the cysteines forming the coordination environment in the clusters and, with a redox potential of <-340mV, the clusters can be readily oxidized by a number of mild cellular oxidants including selenium compounds.[6, 32] In the absence of redox agents, MT typically releases only one of its Zn atoms to an apo-enzyme.[11] Not surprisingly, this atom appears to originate from the more kinetically labile three-Zn cluster of the β-domain.[36] However, in the presence of GSSG, MT can increase both the rate of Zn transfer and the number of atoms ultimately transferred.[11] The oxidized form of the tripeptide appears to form a complex with the MT since evidence of ligand-exchange has been provided by collisionally induced dissociation studies using Q-TOF-MS, which indicate that the multiple carboxylate groups of GSSG form a bimolecular complex with MT that permit co-chelation and subsequent extraction of Zn.[13] GSH, the reduced form of the tripeptide, has the converse effect and prevents Zn release in the absence of GSSG but can potentiate the GSSG induced release of the metal when present at low concentrations.[11]

Under the current experimental conditions, MT-1 preferentially donates approximately twice as much Zn to apo-AP as MT-2. This transfer is quantitatively consistent with the restoration of enzyme activity providing independent confirmation of the accuracy of the technique. These findings are consistent with those obtained by Winge and Miklossy[39] who also noted an approximate 2 fold increase in Zn donation to apo-carbonic anhydrase with rat liver MT-1 relative to MT-2 over a 60 minute period and those of Jiang *et al*,[11] who recorded rate constants of 24 and 16 $M^{-1}.s^{-1}$ for the activation of zinc depleted sheep liver sorbitol dehydrogenase by human MT-1 and MT-2 respectively. This difference in the relative metal lability of the two isoforms may account for the preferential donation of Zn from MT-1 to MT-2 observed in this study and presumably reflects the variations in the amino acid composition between the isoforms which, for the rabbit MT isoforms, appears to be restricted largely to the β-domains of the protein and the replacement of an asparagine residue in position 9 of MT-1 for an aspartic acid in MT-2.[40]

4.5 Limitations and Refinements to the Technique

This research is the first experimental attempt to trace the intermolecular exchange of three distinct Zn sources between three different proteins. While the described technique can provide various insights into the function of MT in Zn homeostasis, it does have a number of inherent limitations. First, recoveries from HPLC columns can be varied, making mass balance calculations for stoichiometric determinations difficult. These problems are exacerbated by substitutive exchanges of metals between the eluting proteins and the column packing material can occur during chromatography, especially when small amounts of protein are injected.[21] Another problem with the technique as described is that the selectivity of the anion-exchange separation procedure is relatively poor. Thus, although the MT isoform preparations used in the present study were purified

by reverse phase HPLC prior to isotopic labeling, we are cognizant that the MT proteins designated as distinct isoforms probably represent a pool of different sub-isoform gene variants with differing affinities for Zn. Based on the elution and retention profiles provided by Sanz-Nebot *et al*[40] for rabbit liver metallothionein from the same vendor, we can tentatively identify the MT-1 used in this study as probably containing isoforms MT-1a and MT-2d and a non-acetylated MT-2d and the MT-2 pool comprising a mixture of subisoforms MT-2a, MT-2b and MT2c. The appearance of MT-2d in the MT-1 preparation is not unexpected since this isoform has an overall global charge distribution comparable to MT-1a.[40] Better selectivity with shorter retention times could be obtained by replacing the conventional DEAE HPLC column used in the current study with a FPLC Mono Q HR 5/5 columns (Pharmacia Biotech, Upsala, Sweden) which has been shown to readily separate the MT-1 and MT-2 samples (Sigma) into at least 4 MT-1 subisoforms and MT-2 into an additional 3 subisoforms.[41] Improved selectivity can also be obtained using a capillary electrophoresis (CE)-interfaced ICP-MS, which has been used to successfully separate different variants of MT isoforms via capillary zone electrophoresis using microconcentric nebulizers prior to ICP-MS detection.[42-45] Similarly, non-denaturing microbore reverse phase applications have been used to separate metal containing isoform variants of MT[42] in conjunction with direct injection high efficiency nebulizer systems to increase both sensitivity and selectivity by reducing the peak broadening of the resolved analyte peak commonly experienced during passage through the spray chamber.[46] Another severe limitation of the current technique is that it is element rather than molecule specific. Consequently, it cannot identify the ligands in the reaction (other than by retention time and UV/VIS properties) and apo-proteins are essentially "transparent" and cannot be detected by the procedure. For example, it is apparent that MT-1 donates significant quantities of Zn during the various reactions. The absence of other metals such as Cu, Ag and Hg that bind avidly to MT is presumably indicative that the protein exists in a metal depleted form after donating its Zn, but confirmatory analyses have yet to be conducted using other technologies. In this respect, unlike electro- or nanospray MS, that can provide information on the degree of metal saturation of AP, MT and other proteins capable of binding more than 1 atom of metal, the use of an ICP-MS detector can provide only an aggregate value of the metal content for the entire population of molecules within the eluting protein peak. However, from both a mechanistic and functional perspective it is important to not only determine the degree of Zn saturation within the protein population but also identify the structures involved in the exchange processes.

4.6 Future Perspectives

Despite the limitations mentioned above, the described technique has a number of distinct virtues for studying metallothionein isoform function. At this point in time, very little is known about the functional differences between the various MT isoforms and subisoforms. Differential expression studies with various transcriptional inducers using conventional reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR have provided tantalizing evidence for specific patterns of gene expression that have been used to indirectly ascribe functionality.[24] However, without simultaneous information on metal composition, loading and movements these inferences must still be considered speculative since the functionality of these proteins, other than perhaps in reactive oxygen species scavenging, is thought to be implicitly tied to their metal content. Given the ubiquity and polymorphism of this super-gene family, and the

pleiotrophic functions that have been ascribed to the group as a whole, it is clear that techniques must be devised that can directly look at the interactive roles of the specific MT variants in Zn distribution within the cell. Biological zinc complexes are silent to the majority of physiochemical techniques used to probe the kinetics of metal/protein complexes other than mass spectroscopy.[12] With further refinement and with the use of multi-micro-bore column switching technologies, the described technique probably has the selectivity to resolve complex transfer processes involving mixtures of proteins in cytosolic matrices. It has good sensitivity, is multi-elemental, has relatively good isotopic precision and can tolerate high salt concentrations that can cause problems for CE and ESI MS applications. The technique is robust, does not require a specialized interface and is easy to use. It therefore adds significantly to the growing repertoire of hyphenated techniques used for metal speciation in cellular systems and,[47] when used in combination with a molecule specific detector, has great potential for studying the essential and toxic roles of metals in life processes.

5 ACKNOWLEDGEMENTS

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